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Impacts of acute hypoxia on the short-snouted seahorse metabolism and behaviour

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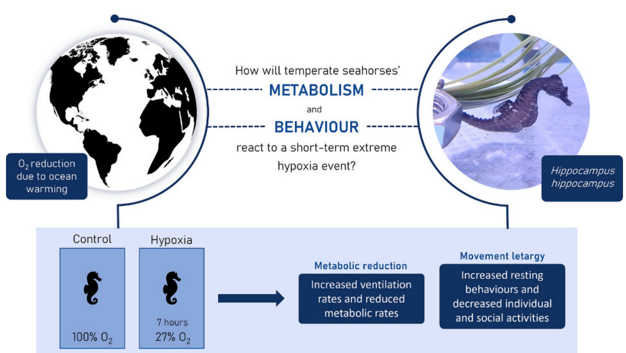
HIGHLIGHTS

- Due to their specific lifestyle, seahorses are sensitive organisms to environmental changes.
- Metabolism, behaviour patterns and food intake of *Hippocampus hippocampus* were evaluated under a hypoxic event.
- The extreme hypoxia caused a decrease in seahorses' metabolism and an accentuated decrease in their activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Seahorses are one of the most unique and enigmatic animals, recognized as flagship species for several conservation issues. Unfortunately, seahorses' populations have been declining and their unique lifestyle may constrain the ability of these animals to evolve in the future climate scenarios. They inhabit shallow coastal waters that display daily or seasonal environmental fluctuations. Yet, few studies have scrutinized the impacts of climate changes on these iconic species. Within this context, the objective of this work was to test the effects of an extreme hypoxia exposure (~27 % dissolved oxygen for approximately 7 h) on the metabolism, behaviour and food intake of the temperate seahorse *Hippocampus hippocampus*. Regarding metabolism, hypoxia exposure led to a significant reduction in metabolic rates and an increase in ventilation rates. Seahorses showed signs of movement lethargy under oxygen depletion. The results show that a small but extreme exposure to hypoxia is tolerable by seahorses despite inducing metabolic and behavioural changes, that may jeopardize the future development and survival of these iconic organisms.

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1. Introduction

Since the industrial revolution, the anthropogenic emissions of greenhouse gases led to unnatural changes in the Earth's thermal balance and, consequently, to atmospheric and ocean warming (Duarte, 2014; Harley et al., 2006; IPCC, 2022). As a result, the frequency, strength, and extent of extreme events are and may continue to increase, such as extreme hypoxia events (Altieri and Gedan, 2014; Diaz and Breitburg, 2009; Diaz and Rosenberg, 2008; IPCC, 2022). Oxygen depletion represents one of the greatest environmental challenges for marine organisms (Earle et al., 2018; Huang et al., 2018; Sampaio et al., 2021; Borges et al., 2022).

In marine and estuarine systems, extreme hypoxia events are characterized by a low level of dissolved oxygen (DO), normally $\leq$2 mg of O_2 L^{-1} (the exact concentration is organism-specific; Breitburg et al., 2018; Diaz and Breitburg, 2009). In coastal and shallow waters, these events occur naturally, due to 1) the strong vertical stratification that limits the exchange of oxygen (O_2) between the different layers of water, 2) the imbalance between respiration and photosynthesis, especially during the night, 3) the low tide, which forms tide pools in the intertidal zones and 4) other specific circumstances like rain and storms, since they change the water flows and/or block water-air oxygen exchange (Altieri and Gedan, 2014; Breitburg et al., 2018; Diaz and Breitburg, 2009). These hypoxic conditions can also be caused or accentuated by eutrophication, which currently is often a result of anthropogenic processes (Diaz and Rosenberg, 2008).

The low O_2 concentration, which can lead to its rapid consumption by aquatic organisms, coupled with global warming, which further reduces the O_2 solubility, can have serious impacts on animals' metabolism and behaviour (Domenici et al., 2017; Willmer et al., 2005; Xing et al., 2014). In normoxic conditions, >95 % of the oxygen consumed by a fish is used in aerobic processes to produce the energy needed by the organisms' physiological processes (Richards, 2009). Upon exposure to hypoxia, fish survival depends on the: 1) fast reorganization of the biochemical and physiological systems to maximize the oxygen uptake rates and sustain the routine metabolic rate or 2) cellular modifications to produce energy in O_2-limiting conditions, through anaerobic processes (Richards, 2009). In turn, this can result in certain changes in fish behaviour, such as in their swimming, feeding, and reproduction, since the energy is directed to more O_2-sensitive tissues, as reviewed by Farrell and Richards (2009).

Seahorses are teleost fish with unusual anatomical and behavioural characteristics, that make them one of the most unique and enigmatic animals (Curtis, 2006; Foster and Vincent, 2004). Unfortunately, seahorses' populations have been declining, mainly due to anthropogenic activities (e.g Harasti, 2016; Martin-Smith and Vincent, 2005; Pierri et al., 2021). Their unique lifestyle, namely, their reduced swimming ability, small distribution, high site fidelity, low batch fecundity, and monogamy, may constrain their ability to adapt and evolve in future climate scenarios (Curtis et al., 2017; Faleiro et al., 2015; Foster and Vincent, 2004). They spend most of their time resting, attached by their tail to holdfasts such as seagrass (Lourie et al., 2004), which also allows them to camouflage and wait motionless for the prey to come close to their snout (Foster and Vincent, 2004). This high site fidelity decreases the chances of these animals finding suitable habitats (Foster and Vincent, 2004). Furthermore, migration can jeopardize the continuity of generations due to the possible breaking of the monogamous pair bond (Faleiro et al., 2015; Foster and Vincent, 2004).

Although several fish species have been shown to be affected by oxygen depletion (e.g. Richards, 2009; Ekau et al., 2010; Wang et al., 2016), few studies have analyzed the effects of hypoxia on the Syngnathidae family (Braga Goncalves et al., 2016; Negreiros et al., 2011; Ripley and Foran, 2007). The tropical seahorse *Hippocampus reidi* showed damage in the DNA strands after an eight-hour exposure to 1.5 mg O_2 L^{-1}, but the mechanisms that led to this change are not yet known. Additionally, an elongation of the gill lamellae was found in those animals, possibly to maximize oxygen uptake from the water (Negreiros et al., 2011). Furthermore, two species of pipefish, *Syngnathus fuscus* and *S. floridae*, tolerated oxygen concentrations below lethal levels (2 and 1 mg O_2 L^{-1} for one to five days) but reduced their sound production and the associated food intake (Ripley and Foran, 2007).

The species *Hippocampus hippocampus*, that inhabits specific biogenic environments of European shallow coastal areas like estuaries (Curtis et al., 2017; Curtis and Vincent, 2005; Lourie et al., 2004), may become quite vulnerable to future climatic scenarios and extreme events, as other seahorses, which made them a flagship species for climate change issues (Aurélio et al., 2013; Curtis et al., 2017; Faleiro et al., 2015). Studies on their physiological processes are important to predict climatic effects and plan conservation measures for the whole ecosystem (Pörtner and Farrell, 2008). Taking into account the scarce information on this topic, the aim of this study was to test the effects of a short but extreme hypoxia event (~ 27 % DO or 2.1 mg O_2 L^{-1}, approximately 7 h) on seahorses' *H. hippocampus*. We aimed to scrutinize the effects of hypoxia on metabolic rates, ventilation rates, behavioural pattern (namely resting, individual and social activities) and food intake.

2. Materials and methods

2.1. Species collection

Adult *H. hippocampus* were provided by Oceanário de Lisboa (Lisbon, Portugal). The previous generation of these animals was captured in the Sado Estuary (Portugal). From the captive facilities of the Oceanário de Lisboa, nine 7-month-old seahorses (four males and five females, ranging from 0.70 to 3.69 g), born from a not selective breeding, were transported under controlled conditions to the recirculating aquaculture systems (RAS) in the Laboratório Marítimo da Guia (LMG, MARE-UL, Cascais, Portugal), where the experiment took place.

2.1.1. Ethical statement

The present experiments and analysis were reviewed and authorized by the Portuguese Foundation for Science and Technology (FCT) and the Faculty of Science of the University of Lisbon animal welfare body (ORBEA) in accordance with the requirements imposed by the national (Decreto-Lei 113/2013) and EU legislation (Directive 2010/63/EU) on animal protection used for scientific purposes.

2.2. Experimental setup and acclimatization

Upon arrival at LMG, the animals were first acclimatized to the new captive conditions for one month, during which no experimental trials were carried out. Seahorses were kept in similar conditions to the ones described in previous studies (Aurélio et al., 2013; Faleiro et al., 2015) and those found in their natural environment: temperature 17.0 ± 0.3 °C, DO approximately 100 % (7.6 ± 0.1 mg of O_2 L^{-1}), salinity 33 ± 1 ppt and pH 8.0 ± 0.1. Ammonia, nitrites and nitrates concentrations were kept below detected values.

Seahorses were distributed into one semi-opened aquaria system, composed of three 70-L acrylic aquaria (30 × 41 × 60 cm) and a common water outflow tank (sump; Fig. S1 in the Supplementary Material). The system functioned as a treatment, and each aquarium as a replicate, with three animals each. Each aquarium had at least one animal of each sex. Natural seawater (salinity 35 ppt) was pumped directly from the sea, filtered and UV-sterilized, being then pumped from the sump to each aquarium. To adapt the salinity of the water to the needs of these fish, fresh water purified by activated carbon was added daily to the systems. The sump had a filtration system, and each aquarium was continuously renewed by a water drip system also filtered and UV-sterilized. This type of system design, along with daily 20 to 30 % water changes, allowed to maintain water quality. Water temperature (17.0 ± 0.3 °C) was regulated through room temperature and a chiller connected to the sump that allowed the temperature to never rise above the intended value. Oxygen levels were controlled by an air compressor connected to air diffusers, three in the sump and one in each aquarium (but with a lower diffusion rate). Aquaria illumination was provided through overhead fluorescent lighting, with a photoperiod of 14 h of light:10 h of dark cycles. Environmental enrichment structures, e.g., artificial plants, plastic chains and nets, were also added to all aquariums to provide holdfast for seahorses' attachment and to improve the welfare of the animals during the experiments (Aurélio et al., 2013; Faleiro et al., 2015). The fish were fed ad libitum, three to five times a day, except the day before the experimental tests. Their diet consisted mainly of frozen food: more frequently and in greater quantity *Mysis* (Fig. S2 in the Supplementary Material), and less frequently and in smaller quantities enriched adult *Artemia* and copepods. Live *Mysis* were introduced as much as possible.

Seahorses were then exposed to two different oxygen level conditions:

1) control, representing the current annual mean environmental conditions of the Sado estuary (~ 100 % DO ~7.6 mg O_2 L^{-1}, $n = 8$ in the metabolic rates, $n = 9$ in the ventilation rates and behavioural patterns); and
2) hypoxia, simulating an extreme decrease in DO in the water (~ 27 % DO ~2.1 mg O_2 L^{-1}, $n = 7$ in the metabolic rates, $n = 8$ in the ventilation rates and behavioural patterns).

To apply refinement and reduction of the 3 R's rules of the ethics of animal experimentation, first introduced by Russell and Burch (1959), individuals from hypoxia treatments were also used under control conditions after a week where no experimental trials were performed.

2.2.1. Hypoxia exposure

A nitrogen (N_2) gas injection was used to regulate and maintain the DO around 27 % (2.1 mg O_2 L^{-1}). To make sure that the N_2 was sufficient to maintain the desired levels of O_2 during exposure, an Ardoxy controlling system connected to solenoid valves automatically controlled the N_2 flow injected into the water, by injecting N_2 when O_2 levels rise above 27 % (2.1 mg L^{-1}) and stopping injecting when the reverse occurs. The hypoxic exposure had a total duration of approximately 7 h, divided into two parts: 1) 5 h30 of exposure occurred in the chambers during the oxygen uptake rates (MO_2) measurement and 2) approximately 1 h in the aquarium, after the MO_2 measurement. At the beginning of the respirometry trial, a slow decrease in O_2 concentration was implemented in the water that filled the chambers until the desired O_2 concentration inside the chambers was obtained (after an hour, the O_2 levels were at 27 %). After 5 h30 of MO_2 measurement, which includes the time the oxygen depletion was implemented, the fish were again transferred to their aquarium tanks where exposure to hypoxia conditions continued for approximately an hour. To achieve desired dissolved O_2 levels in the aquarium tanks, N_2 was injected into a cylindrical column tank, added to the overall system design, which altered the path of the water in the recirculating system. The water was pumped from the sump to the cylinder column and only then to the aquarium. A scheme of the hypoxia exposure is represented in Fig. S3 in the Supplementary Material.

2.3. Metabolic rates and ventilation rates

Following previous methods (Clark et al., 2013; Paula et al., 2022; Rummer et al., 2016), an intermittent flow respirometry system was used for the measurement of the oxygen uptake rates (MO_2), applied to estimate the routine metabolic rates (RMR). A scheme of the hypoxia exposure is represented in Fig. S4 in the Supplementary Material. At the beginning of the experimental day, seahorses were individually placed in 606 ml (including tygon chemical tubing) respirometry chambers which were then completely closed so that there was no external infiltration of O_2. The chambers were submerged in a recirculating system, in a water bath with the same temperature conditions as the respective treatment, ensured by a digital heater and a chiller, regulated by a Profilux controlling system with a temperature probe. Small holdfasts were provided to the seahorse's attachment. Before each trial, a 24-hour period of starvation was implemented in the experimental aquariums, to guarantee a postabsorptive metabolic state (Niimi and Beamish, 1974). During the entire process, the animals were continuously and carefully observed to ensure their well-being.

The MO_2 were measured through seven cycles of 30 min, each consisting of a measurement period (25 min), a waiting period (1 min) and a flush period (4 min). Each respirometry had a duration of 5 h30, of which 2 h of acclimatization and 3 h30 of O_2 measurements, as described in Aurélio et al. (2013). The initial period of 2 h allowed acclimatization to the new environment and to the desired oxygen concentration in the hypoxia treatments. The total duration of the measurement period ensured that the O_2 levels inside the chambers never went below 80 % air saturation (Paula et al., 2022) in the control treatment, guaranteeing that the measurements of MO_2 are not influenced by any sharp metabolic decrease which may affect animal welfare. Between measurement periods, an automated flush pump submerged in a tank with treatment water supplied and renewed the chambers with clean seawater. These pumps were regulated via Profilux controlling system with a programmed timing sequence. The flush period was long enough to allow the oxygen levels in the chambers to be completely renewed with new treatment water.

To ensure the water mixture inside each chamber during the measurement periods, each chamber was connected to an individual respirometry pump that boosted the water through the chamber in an external close loop of gas-tight tubing (flow rate: 100 ml/min). Connected to this tubing, each chamber had a flow-through cell with an integrated optical oxygen sensor linked with fiber-optic cables to a Firesting Optical Oxygen Meter. These sensors recorded the temperature-compensated oxygen concentration (mg L^{-1}) of the water every 2 s, saving data on a connected portable computer. Before each respirometry trial, the temperature and the O_2 sensors of the setup were calibrated. To eliminate the influence of possible bacteria and microorganisms' activity, before and after each respirometry trial, the entire setup was disinfected with hydrogen peroxide, cleaned with fresh water and then refilled with clean and filtered seawater of each treatment. To further minimize the microorganisms' influence in the MO_2 measurements, a background respiration was performed in each chamber, before and after each run, without the respective seahorse inside. Since it was assumed that background MO_2 increased linearly (from start to end of each run), the results of the background respiration were then subtracted from the respective seahorse respiration. The whole analysis was made with R software package "respR" (Harianto et al., 2019), where the O_2 concentration data was corrected for fish mass [$mgO_2\ g_{fish}^{-1}\ h^{-1}$].

After the 5 h30 of respirometry trials, seahorses were transferred back to their original aquarium, with the same conditions as the treatment, where the experiment continued. After an acclimatization period of 30 min to the aquarium, individual ventilation rates were measured by counting the number of opercular beats per minute. This procedure was repeated three times per individual.

2.4. Behavioural patterns

A careful observation during the initial month of acclimatization to captive conditions allowed the formulation of a behavioural ethogram to *H. hippocampus* (Table 1). The behaviours, observed several times and in several animals, were divided into three categories: 1) rest, which represents the resting behaviours, 2) individual activities, considering the more active behaviours that these animals by themselves, and 3) social activities, which includes active behaviours that involve two or more seahorses.

After the measurement of the ventilation rates, 30-minute videos were recorded to assess seahorses' activity patterns and their food intake

Table 1
Ethogram of *Hippocampus hippocampus* activity patterns. The visual observations made during the initial month of acclimatization were complemented with terminologies from Anderson et al. (2011), Claassens and Hodgson (2018), Faleiro et al. (2008), Felício et al. (2006), Naud et al. (2008), Pimentel et al. (2016), and Vincent (1994).

Category	Behaviour	Description
Rest	Stationary	Seahorse remains completely still.
	Swinging	Light head and/or body movements while the seahorse is attached to a holdfast.
Individual activity	Adjustment	Seahorse often adjusts the tail on the holdfast, rotating or moving vertically along it.
	Slow body movement	Seahorse moves slowly, mainly using the tail and not the dorsal and/or pectoral fins or using them lightly to propel themselves.
	Swimming	Seahorse swims actively, constantly moving the dorsal and pectoral fins.
	Feeding	Seahorse visualizes and approaches the prey, stretching his body while attached to a holdfast or swimming toward it. It directs the snout to the prey and attacks, capturing it or not.
		Capture: When the seahorse catches and ingests the prey.
		Miss: When the seahorse attacks but does not catch the prey.
		Attack: Sum of Capture and Miss behaviours.
Social activity	Interaction with aggression	Seahorses interact via tail wrestling (especially when one of them is trying to free itself from the tail grasp of the other seahorse), snapping (using the snout or other part of the body) and/or chasing.
	Interaction without aggression	Seahorses from the same sex follow each other; swim together around a holdfast, at the bottom of the aquarium tank or in the water column; grab each other's tails or inflated the pouch with water (in males only).
	Courtship	Seahorses from different sex approach, bright their colors, hold each other and promenade, raise in the water column (tilting and quivering), copulate, and transfer the oocyte (or attempt to).

in their original aquarium tanks. The time spent by each seahorse in each category and behaviour was measured and converted into a percentage of total time. At the beginning of the recordings, 20 live *Mysis* were placed in the aquarium and at the 20-minute mark, 10 frozen ones were added. Food intake, both live and frozen *Mysis*, was visually counted in the recordings and transformed into percentages of the total live and frozen *Mysis* amount, respectively. Still regarding the feeding behaviour, the frequencies of attack, capture and miss were recorded, being then calculated as the percentage of the total *Mysis* amount (based on Drost, 1987; Pimentel et al., 2016). Previous studies show that the pacific and careful observation of seahorses does not alter the normal behaviours that these fish exhibit (e.g. Aurélio et al., 2013; Faleiro et al., 2008).

2.5. Data analysis

Statistical analysis of all variables was performed in R (version 4.0.2, R Core Team, 2020), via Generalized Linear Mixed Models (GLMM, Zuur et al., 2009). Treatment was used as a fixed factor and individuals as a random factor, to consider the experimental design and account for dependence between observations in the same individual. The random effect was kept in all models regardless of the amount of variation it explained, as recommended by Barr et al. (2013). All models were tested using the "glmmTMB" function from package "glmmTMB" (Brooks et al., 2017). Function "Anova" from the package "car" (Fox and Weisberg, 2011) was used to perform Type II Wald chi-squared tests of each model, to test the significance of each explanatory variable over the response variable (Tables S1 and S2 in the Supplementary Material). Post-hoc multiple comparisons between treatments were also performed, using the "emmeans" package (Searle et al., 1980), with Tukey corrections to minimize type I error (Tables S3 and S4 in the Supplementary Material; Lenth, 2022). The package "performance" (Lüdecke et al., 2021) was used to validate the models' performance and assumptions.

3. Results

3.1. Metabolic rates and ventilation rates

Regarding routine metabolic rates (RMR, Fig. 1a), hypoxia (H) elicited a significant decrease in the RMR of the adult seahorses ($p < 0.0001$). Hypoxia promoted a significant increase in the ventilation rates (Fig. 1b, $p < 0.0001$), reaching a value of 55.3 ± 2.6 beats min^{-1}, which represents an increase of 129.5 % in comparison to control conditions.

3.2. Behavioural patterns and food intake

3.2.1. Rest

Seahorses under control conditions spent an average of 80.4 ± 2.5 % of their time resting (Fig. 2a), on the other hand, the time they spent resting increased to 98.8 ± 0.5 % under H ($p = 0.0001$), which represented an increase of 22.9 %.

Regarding the stationary behaviour (Fig. 2b), exposure to H prompted significant changes in this behaviour ($p < 0.0001$). The time that the seahorses remained stationary increased from 2.6 ± 1.2 % under control conditions to 80.1 ± 4.4 % in the hypoxia treatment. Alongside, the percentage of time spent by seahorses swinging (Fig. 2c) was 77.1 ± 3.5 under control conditions, which decreased significantly with hypoxia treatment to 17.9 ± 3.4 % under hypoxia (H) ($p < 0.0001$).

3.2.2. Individual activity

Hypoxia treatment did cause a significant change in seahorses' individual activity ($p < 0.0001$), from an average of 15.1 ± 1.8 % under control conditions to 0.8 ± 0.4 % under hypoxia. Following the same trend, the reduction of O_2 led to significant impacts on the slow body movement behaviour (Fig. 3b). The control group of seahorses spent an average of 12.3 ± 1.6 % of their time in this behaviour, which decreased significantly to 0.8 ± 0.3 % under H ($p < 0.0001$). No significant differences were found between treatments regarding swimming (Fig. 3c, $p > 0.05$) and adjustment behaviours (Fig. 3d, $p > 0.05$). Seahorses feeding duration (Fig. 3e), which lasted an average of 1.9 ± 0.5 % under control conditions, decreased significantly to 0.2 ± 0.1 % under hypoxia ($p = 0.0115$).

Regarding live and frozen food intake (Fig. 4a and Fig. 4b, respectively), there was no significant effect on the live *Mysis* consumption between control conditions and hypoxia treatments ($p > 0.05$). The percentage of attacks (Fig. 5a), which includes total live and frozen *Mysis*, did not vary significantly with treatments ($p > 0.05$). Regarding the capture success (Fig. 5b), hypoxia caused a significant decrease to 0.5 ± 0.4 preys ingested ($p = 0.0117$). The miss percentage did not follow the same trend since it did not show any significant changes with hypoxia exposure ($p > 0.05$, Fig. S5 in the Supplementary Material).

3.2.3. Social activity

Regarding the social activity of seahorses (Fig. 6a), there was a significant decrease between the control (3.7 ± 1.5 %) and hypoxia treatment (1.3 ± 0.6 %, $p > 0.05$). Moreover, there were no significant changes in relation to interaction without aggression and courtship behaviours among treatments (Fig. 6b and c, $p > 0.05$).

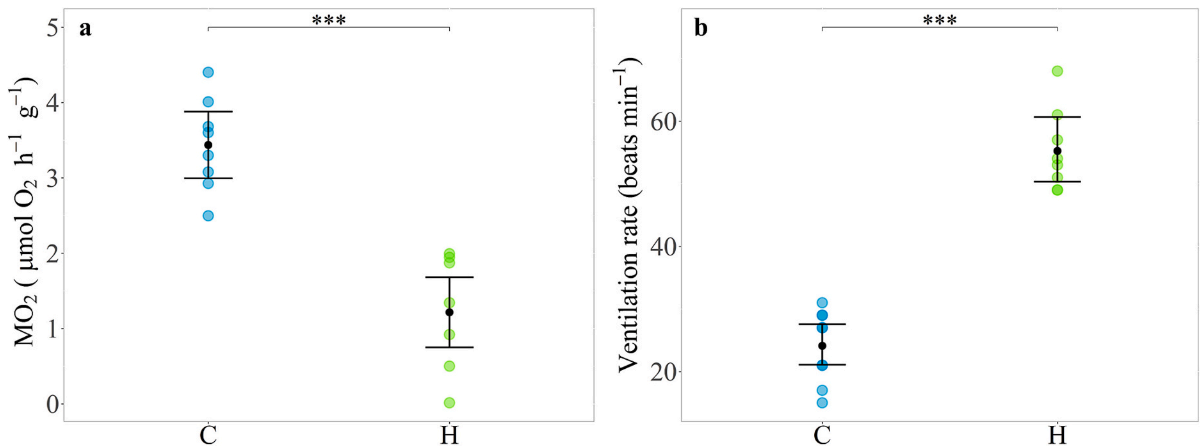

Fig. 1. a) Oxygen consumption rates (MO_2) of control (C, $n = 8$) and hypoxia (H, $n = 7$) treatments and b) ventilation rates of control (C, $n = 9$) and hypoxia (H, $n = 8$) treatments of seahorses *Hippocampus hippocampus* exposed to the control (C, $n = 8$) and hypoxia (H, $n = 7$) treatments. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (p-value $< \alpha$, $\alpha = 0.05$) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

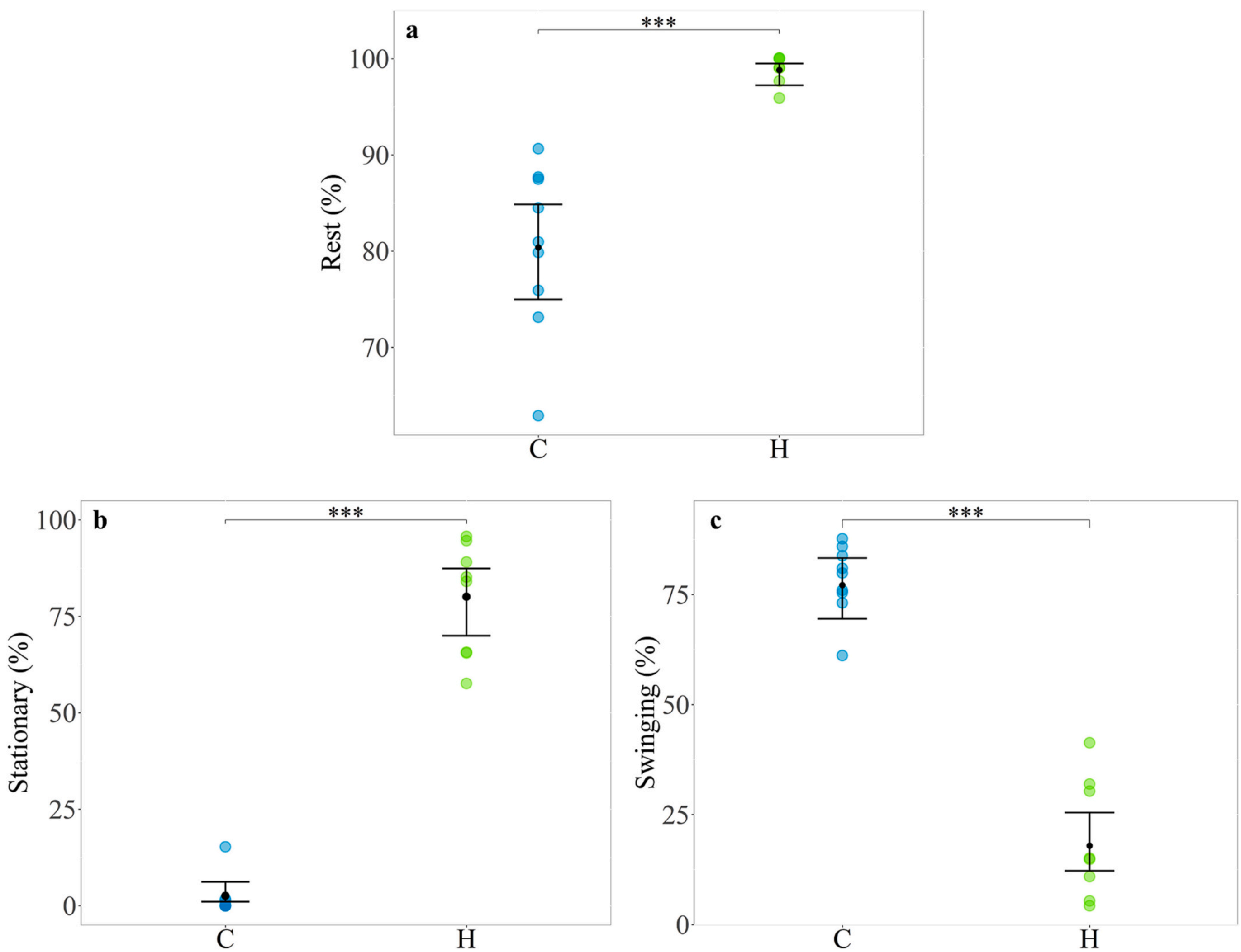

Fig. 2. Behavioural patterns of seahorses *Hippocampus hippocampus* exposed to the control (C, $n = 9$) and hypoxia (H, $n = 8$) treatments. a) Rest category, which includes b) stationary and c) swinging behaviours. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (p-value $< \alpha$, $\alpha = 0.05$) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

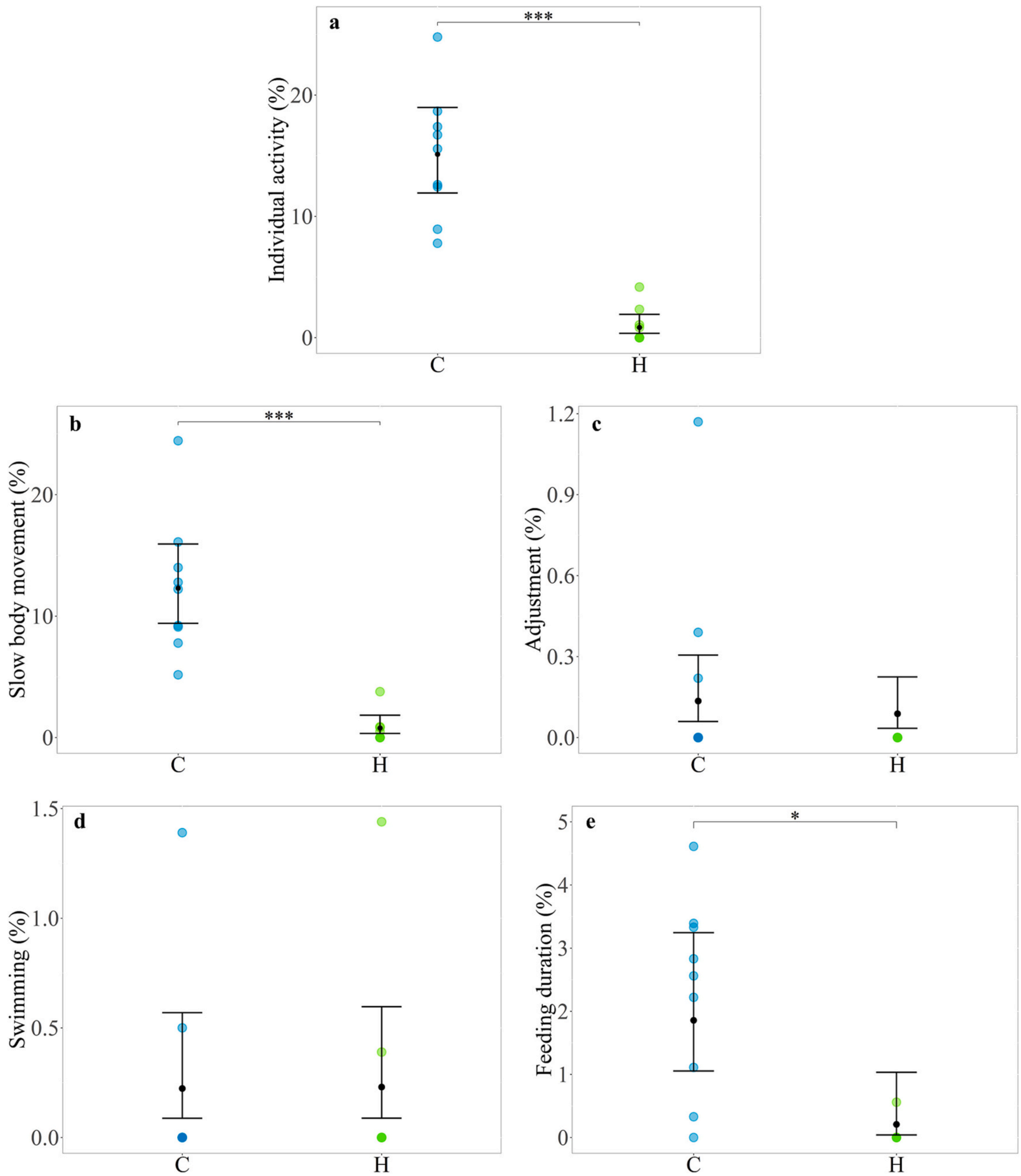

Fig. 3. Behavioural patterns of seahorses *Hippocampus hippocampus* exposed to the control (C, $n = 9$) and hypoxia (H, $n = 8$) treatments. a) Individual activity category, which includes b) slow body movement, c) swimming, d) adjustment and e) feeding behaviours. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (p-value $< \alpha$, $\alpha = 0.05$) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

4. Discussion

Currently, little is known about the path that climate change will take and how it will influence marine organisms and ecosystems. When considering coastal areas, the need for information is very important due to their ecological and socio-economic importance, and the fact that oxygen depletion is being felt at a faster rate (Ainsworth et al., 2019; Frölicher and Laufkötter, 2018; Mackenzie and Schiedek, 2007). Like many other coastal organisms, seahorses have developed physiological and behavioural adaptation mechanisms to survive in shallow coastal areas like estuaries, that undergo seasonal and daily environmental changes (Coelho et al., 2023; Pigliucci, 2003; Mascaró et al., 2016). Yet, the present study shows that even though they exhibit mechanisms that make them able to tolerate a short period of an extreme hypoxia event (approximately 27 % DO or 2.1 mg O_2 L^{-1}), some biological disruptions were reported here.

The hypoxia exposure resulted in a significant decrease in the routine metabolic rates of *H. hippocampus*. This response may be a coping strategy to facilitate the tolerance to future extreme hypoxic events that are expected to be more frequent in the shallow seagrass meadows of estuaries (Schmidt et al., 2019; Sampaio et al., 2021), where minimal oxygen concentrations already occur sporadically due to overnight respiration (e.g. Cravo et al., 2020; Newton et al., 2010). To tolerate such environmental stressful and extreme conditions, some physiological processes such as metabolic depression and the production of biochemical markers associated with anaerobic metabolism may occur to help fish overcome the hypoxic conditions (Abdel-Tawwab et al., 2019; Hochachka and Somero, 2002; Storey and Storey, 2004; Richards,

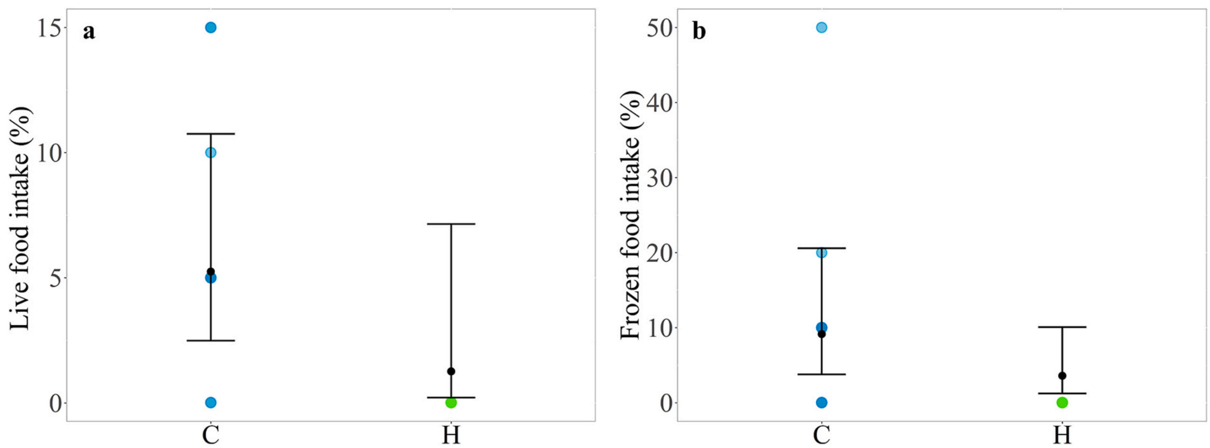

Fig. 4. Food intake of seahorses *Hippocampus hippocampus* exposed to the control (C, *n* = 9), and hypoxia (H, *n* = 8) treatments. a) Percentage of live *Mysis* ingested. b) Percentage of frozen *Mysis* ingested. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (*p*-value < α, α = 0.05) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

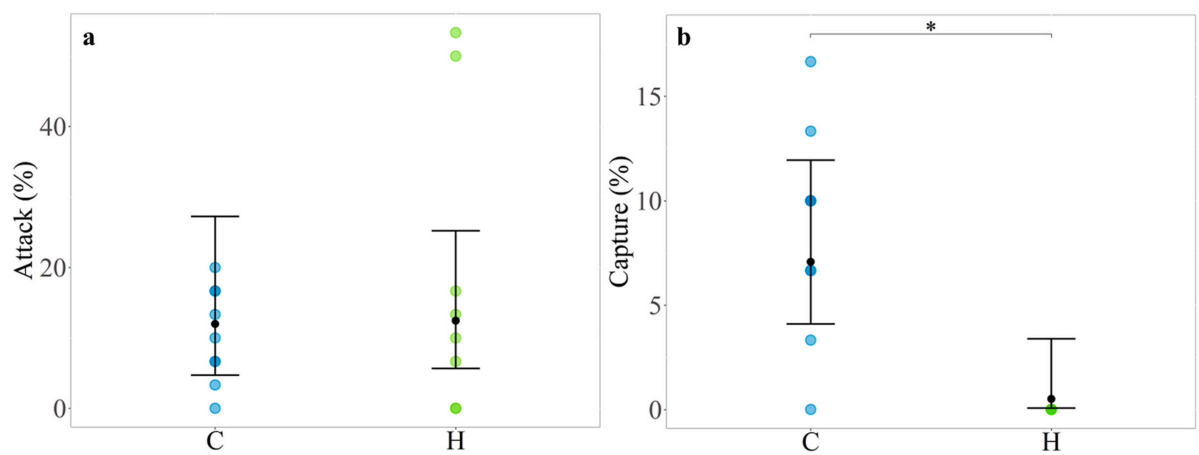

Fig. 5. Feeding behaviour patterns of seahorses *Hippocampus hippocampus* exposed to the control (C, *n* = 9) and hypoxia (H, *n* = 8 treatments. a) Percentage of attacks and b) capture behaviours. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (*p*-value < α, α = 0.05) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

2009; Wu, 2002), however they are likely to be unsustainable on longer timescales. Thus, it is crucial that further studies scrutinize the effects of hypoxia in common regulatory responses and metabolic pathways that may minimize the oxygen depletion consequences, to understand what the metabolic costs are to sustain basic maintenance and the severity of distress in the seahorses. In the future, this will help to comprehend whether changes will occur in the fitness of the animals, and to what extent this may jeopardize the organism and possibly population development and survival (Vaquer-Sunyer and Duarte, 2008; Sampaio et al., 2021). Associated with the metabolic decrease, an increase in opercular beats was observed in the present study. Changes in respiratory patterns, such as increased ventilation and heart rates, and even changes in the oxygen binding capacity of hemoglobin are some of the first possible physiological responses of some species during periods of oxygen depletion (Wu, 2002) to avoid tissue damage. This allows to maximize the O_2 extraction from the environment and maintain the oxygen delivery (Wu, 2002). Negreiros et al. (2011) even detected an elongation of the gill lamellae on *Hippocampus reidi* exposed to extreme hypoxia, probably associated with a higher blood circulation in the gills, that allows a greater gas exchange efficiency.

Seahorses have reduced swimming abilities, spending most of their time resting attached to an holdfast (Foster and Vincent, 2004; Lourie et al., 2004). Despite that, in the present study, hypoxia exposure significantly increased the time seahorses spent resting, showing signs of movement lethargy. Furthermore, the time seahorses spent resting was divided and analyzed into two different behaviour categories, as they stayed completely stationary (not active at all) or slightly moving their heads, the so-called swinging behaviour. With the results of these two behaviours, we can verify that the fish in hypoxia significantly reduced the time they spent swinging their heads since they chose to remain completely stationary. Furthermore, despite being less frequent, seahorses also have individual activities that involve their movement, from simple adjustments or small movements between holdfasts, to active swimming and feeding (e.g. Aurélio et al., 2013; Faleiro et al., 2008; Faleiro et al., 2015). Regarding this individual activity, except for adjustment and swimming behaviours, there was a considerable impact of the hypoxia event, which again resulted in much less active animals. In addition to individual activities, *H. hippocampus* also presents social activities, between the same sex or between different sexes, in this case considered as reproduction behaviours. In the present study, there was only a decrease in the overall social activity category, but no effects were detected on its specific behaviours (i.e. courtship and interaction with/without aggression), which may have occurred due to the reduced number of observations and/or the short period of analysis. In fish, when the physiological strategies to face oxygen depletion are no longer sufficient to maintain the normal functioning of the metabolism, the basic

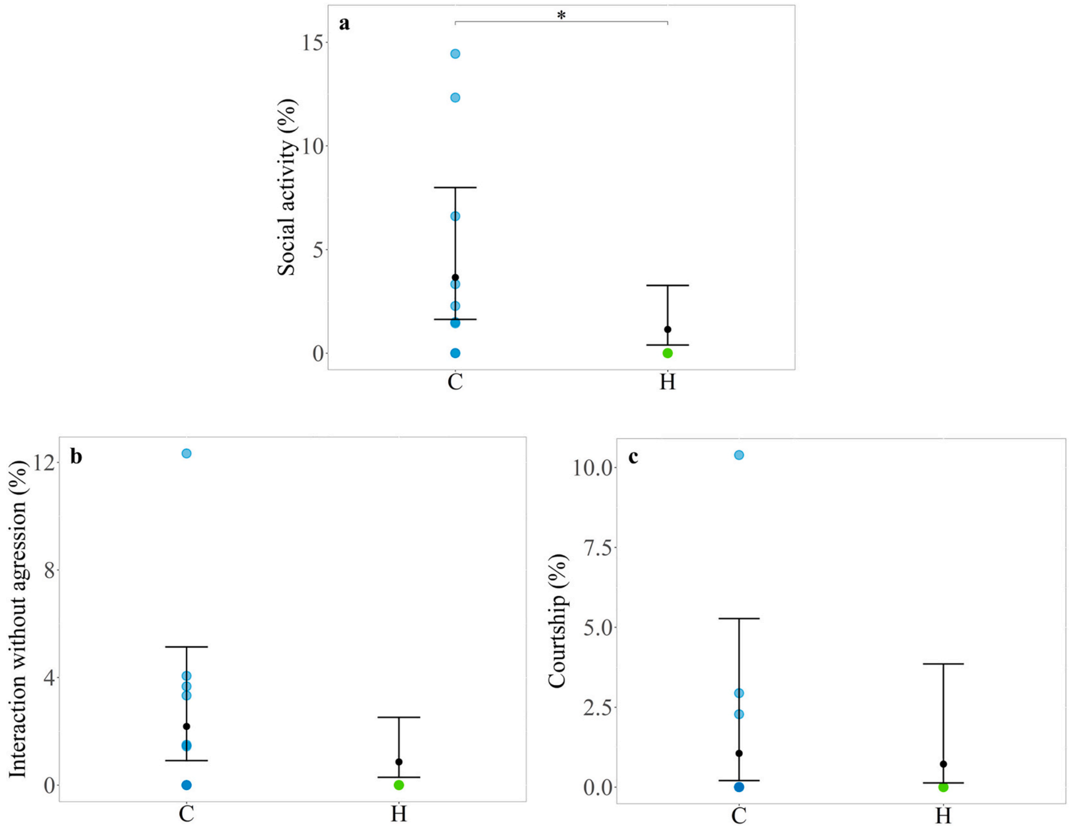

Fig. 6. Behavioural patterns of seahorses *Hippocampus hippocampus* exposed to the control (C, $n = 9$) and hypoxia (H, $n = 8$) treatments. a) Social activity category, which includes b) interaction without aggression and c) courtship. Solid black points correspond to predicted means ±95 % confidence intervals. The dots with different colors represent the observations for each treatment (blue for control and green for hypoxia). Significant differences (p-value $< \alpha$, $\alpha = 0.05$) are represented as: * < 0.05, ** < 0.01 and *** < 0.001.

maintenance activities may be reduced and certain behavioural adjustments can be made, such as reducing their activity level (Chapman and Mckenzie, 2009). This behavioural flexibility may allow energy to be allocated from non-essential processes to essential maintenance costs (Chapman and Mckenzie, 2009; Claireaux and Chabot, 2016). This general reduction in the activity level has already been observed in other fish species, such as the Eurasian perch (Douxfils et al., 2012), which remained static at the bottom of the tank, possibly as a way to save energy (Abdel-Tawwab et al., 2019; Douxfils et al., 2012). Furthermore, it is expected that fish that can experience periods of hypoxia in their habitat and have a relatively sedentary lifestyle will further reduce their activity (Chapman and Mckenzie, 2009; Domenici et al., 2013). Thus, these lethargic responses may indicate that seahorses were struggling against the hypoxic event, however, it remains unclear if they are able to tolerate long-term exposure or more frequent extreme hypoxic events, and what would be the long-term consequences of this lethargic state under hypoxia. Ultimately it may affect their growth, development and reproductive performance, and lately population dynamics and survival.

Regarding seahorses' feeding behaviours, extreme hypoxia led to a significant decrease in the feeding duration and the capture success, which is possibly related to the reduced seahorses' activity and locomotion, which limits the capture and pursuit of prey (Wu, 2002). This result was also observed in two pipefish species *Syngnathus fuscus* and *S. floridae* within one day of extreme hypoxic conditions (Ripley and Foran, 2007). Other fish species, like the spotted wolffish, rainbow trout, turbot, and European sea bass, also reduced their food consumption when exposed to different levels and periods of low dissolved oxygen (Foss et al., 2002; Glencross, 2009; Pichavant et al., 2001). Since feed intake is correlated with fish growth (Magnoni et al., 2018), these results may imply risks for the performance, reproduction and welfare of *H. hippocampus*. Furthermore, the reduction of predation can cause disturbing impacts on the dynamics of communities and consequently on the ecosystem due to changes in food availability (Ripley and Foran, 2007).

5. Conclusion

Summing up, a short but extreme period of oxygen depletion, despite appearing tolerable, resulted in a metabolic decrease, coupled with a reduction in the seahorse *H. hippocampus* activity and feeding. In the long run, either seahorses are able to adapt to such extreme events or these findings may imply a cascade of consequences due to changes in the fitness and development of these animals and due to the larger susceptibility to other factors such as predation, starvation and diseases (Pörtner and Knust, 2007; Wang and Overgaard, 2007). Knowledge of the effects of these events on seahorses is still scarce, but is extremely important as the frequency, strength and duration of these events is increasing and is expected to worsen if current climatic conditions continue (Altieri and Gedan, 2014; Burger et al., 2020; IPCC, 2022; Oliver et al., 2018). More studies are still needed to elucidate the effects of extreme environmental conditions and how animals may endure under such conditions. Thus, future studies should focus on the individual and combined impacts of climate change on the various species of seahorses, considering both long-term environmental changes such as warming, acidification and deoxygenation, as well as short-term extreme temperature, acidification and hypoxic events. In addition, it will be relevant to understand how the different life stages of these animals will react to climate changes in order to understand the possibility of acclimatization and adaptation of the different generations.

CRediT authorship contribution statement

Matilde Gomes: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Vanessa M. Lopes:** Methodology, Software, Resources, Investigation, Writing – review & editing. **Monica G. Mai:** Methodology, Investigation, Writing – review & editing. **José R. Paula:** Methodology, Software, Resources, Writing – review & editing. **Regina Bispo:** Methodology, Formal analysis, Software, Writing – review & editing. **Hugo Batista:** Resources, Writing – review & editing. **Catarina Barraca:** Resources, Writing – review & editing. **Núria Baylina:** Resources, Writing – review & editing. **Rui Rosa:** Conceptualization, Validation, Formal analysis, Resources, Investigation, Writing – review & editing, Supervision, Project administration, Visualization. **Marta S. Pimentel:** Conceptualization, Methodology, Validation, Software, Formal analysis, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166893.

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